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2-Amino-2-deoxyisochorismate Is a Key Intermediate in *Bacillus subtilis* *p*-Aminobenzoic Acid Biosynthesis

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Among biosynthetic pathways, chorismate is an important and central branch-point metabolite in the biosynthesis of the aromatic amino acids tryptophan (Trp), tyrosine (Tyr), and phenylalanine (Phe) as well as *p*-aminobenzoic acid (*p*ABA), a constituent of the vitamin folic acid.[1] As the enzymes of *p*ABA and folic acid biosynthesis occur in many microorganisms, parasites, and plants but not in humans, *p*ABA biosynthesis is an interesting target for antibiotics and pesticidal and herbicidal agents. Prominent synthetic inhibitors of the tetrahydrofolate pathway are sulfonamides and trimethoprim,[2] and the first natural-product inhibitors of *p*ABA biosynthesis, atrop-abyssomicin C and abyssomicin C, have recently been described by our group.[3]

A selection of chorismate-utilizing enzymes, including 4-amino-4-deoxychorismate synthase (ADCS), isochorismate synthase (IS), and anthranilate synthase (AS), have been suggested to be evolutionarily related on the basis of sequence homology, structural similarity, and a unified reaction mechanism (Figure 1).[4] In all three enzymatic reactions, the C4 hydroxy group of chorismate is replaced by addition of a nucleophile at the C2 position. IS and AS use water and ammonia, respectively, as the nucleophile, whereas in the case of ADCS, the ε-amino group of lysine 274 transiently binds to C2 of chorismate.[5] In the AS-catalyzed reaction, the 2-amino-2-deoxyisochorismate (ADIC) intermediate is not released from the enzyme. Instead, pyruvate is eliminated to yield anthranilate (*o*ABA). In contrast, IS releases its product isochorismate, and in the ADCS reaction, ammonia is added to the covalent enzyme intermediate, which is then converted to 4-amino-4-deoxychorismate (ADC).[5] ADC subsequently undergoes an elimination of pyruvate catalyzed by ADC lyase (ADCL) to produce *p*ABA. ADCS itself is a heterodimer composed of two nonidentical subunits, PabA and PabB. PabA functions as a glutamine amidotransferase that hydrolyzes glutamine (Gln) to glutamate (Glu) and NH_4^+. PabB catalyzes the formation of 4-amino-4-deoxychorismate from chorismate and ammonia provided by the amidotransferase PabA under retention of the configuration. This reaction mechanism of ADCS has been demonstrated for *Escherichia coli* to date.[4,5] Furthermore, a crystal structure for *E. coli* PabB was solved in 2002 by Parsons et al.[6] Comparative sequence analysis of the *E. coli* and *Bacillus subtilis* ADCS PabB subunits (33% identities, 52% positives) revealed that the catalytically active amino acid residue K274 of the *E. coli* enzyme corresponds to A283 in *B. subtilis*, and the question arose whether the enzymatic mechanism would be the same for the two enzymes.

In a first step, we therefore investigated the ability of *B. subtilis* PabB to form a covalent intermediate with chorismate, as reported for *E. coli* PabB. Using an electrospray mass spectrometry (ESI-MS)-based approach described by the groups of Abell and Toney,[5] we analyzed and compared *E. coli* and *B. subtilis* PabB before and after incubation with chorismate. For *E. coli* PabB incubated with chorismate, we detected two signals in the mass spectrum with a mass difference of 208 Da (Figure 2A). The second signal

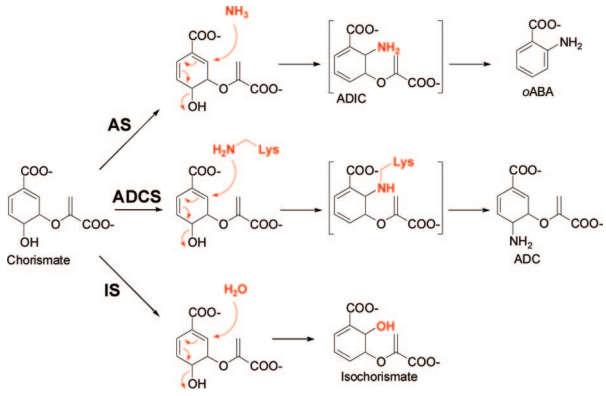

Figure 1. Three chorismate-utilizing enzymes (*E. coli*) sharing a common reaction mechanism: anthranilate synthase (AS), 4-amino-4-deoxychorismate synthase (ADCS), and isochorismate synthase (IS) all replace the C4 hydroxy group of chorismate by addition of a nucleophile at the C2 position.

diminished upon addition of NH_4^+ and did not appear if chorismate was not added to the reaction mixture. Therefore, this signal corresponds to the enzyme-bound chorismate intermediate described previously.[5] For *B. subtilis* PabB, however, incubation with chorismate yielded a single peak, strongly suggesting that no covalent adduct with chorismate was formed (Figure 2B). In an attempt to convert *B. subtilis* ADCS to *E. coli* ADCS, an A283K mutant of *B. subtilis* PabB was generated. The mutant enzyme was analyzed by ESI-MS before and after incubation with chorismate as well. No covalent adduct formation was observed, however, as incubation with chorismate yielded a single peak (Figure 2C). In addition to this finding, the A283K mutant of *B. subtilis* PabB showed no activity in enzyme assays. As we assumed that steric hindrance could be the reason, with the lysine residue being too large within the active site for effective catalysis, two additional mutants of *B. subtilis* PabB, A283V and A283I, were generated. In choosing these mutants, we considered the decreasing steric demands of the corresponding amino acid residues (A283I > A283V). While the A283I mutant completely lacked activity as well, a marginal residual activity was detected for the A283V mutant, confirming our hypothesis that steric reasons account for the lack of activity of the A283K mutant of *B. subtilis* PabB.

The fact that the crucial step of covalent adduct formation of chorismate and *E. coli* PabB was not observed for *B. subtilis* PabB confirmed our initial assumption that their catalytic mechanisms might be different.

Subsequent HPLC−MS analyses of reaction mixtures supported the hypothesis of different reaction mechanisms for *E. coli* ADCS and *B. subtilis* ADCS (Figure 3). As expected, the reaction of *E. coli* ADCS with chorismate in the presence of NH_4^+ or Gln yielded ADC (*m*/*z* 226) as the only product (Figure 3A). Upon addition of *E. coli* ADCL, *p*ABA (*m*/*z* 138) was formed (Figure 3D). In

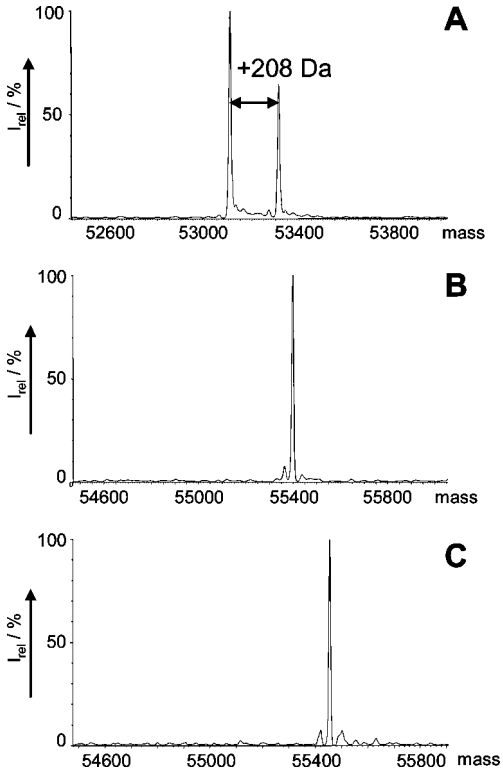

Figure 2. Charge-deconvoluted ESI-qTof mass spectra of ADCS after incubation with chorismate. (A) *E. coli* ADCS + chorismate incubated for 15 min. A mass shift of 208 Da is observed, corresponding to formation of a covalent chorismate intermediate. The mass of unmodified PabB is 53105 Da. (B) *B. subtilis* ADCS + chorismate incubated for 15 min. No covalent adduct is formed. The mass of unmodified PabB is 55399. (C) *B. subtilis* ADCS A283K mutant + chorismate incubated for 15 min. No covalent adduct is formed. The mass of unmodified PabB A283K mutant is 55456.

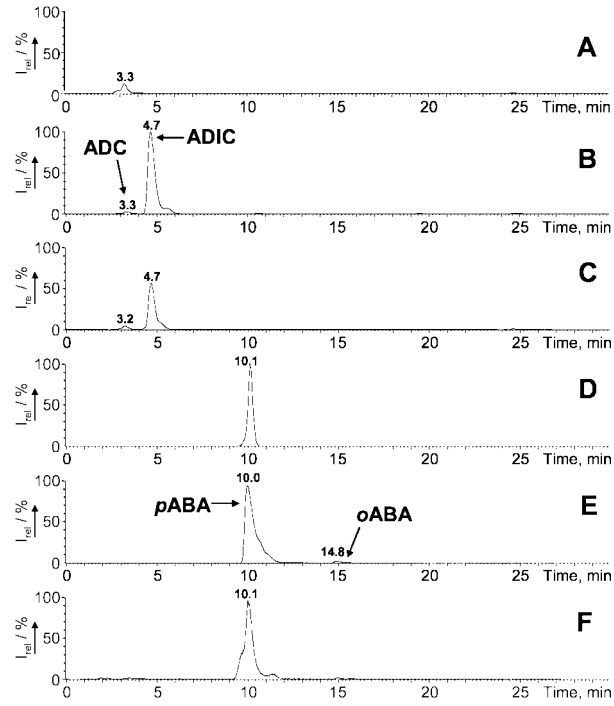

Figure 3. (A−C) HPLC−MS chromatograms (extracted ion *m*/*z* 226) of reaction mixtures: (A) *E. coli* ADCS + chorismate + NH_4^+; (B) *B. subtilis* ADCS + chorismate + NH_4^+; (C) *E. coli* K274A ADCS + chorismate + NH_4^+. (D−F) HPLC−MS chromatograms (extracted ion *m*/*z* 138) of reaction mixtures: (D) *E. coli* ADCS + ADCL + chorismate + NH_4^+; (E) *B. subtilis* ADCS + ADCL + chorismate + NH_4^+; (F) *B. subtilis* ADCS + ADCL + ADIC + NH_4^+. *p*ABA and *o*ABA were identified on the basis of retention times and characteristic MS/MS spectra by comparison to synthetic standards.

contrast, the *B. subtilis* ADCS reaction generated two compounds with *m*/*z* 226, the ADC peak (t_R = 3.3 min) and a prominent second peak (t_R = 4.7 min) (Figure 3B). In addition, *o*ABA (*m*/*z* 138, t_R = 14.8 min) could be identified by comparison to a synthetic standard as a byproduct in the *B. subtilis* ADCS reaction. Addition of *B. subtilis* ADCL to this reaction mixture led to the formation of *p*ABA (*m*/*z* 138, t_R = 10.0 min) (Figure 3E). The *o*ABA peak (*m*/*z* 138, t_R = 14.8 min) in Figure 3E results from the ADCS-catalyzed reaction and is also present if ADCL is omitted. The main product (t_R = 4.7 min) of the *B. subtilis* ADCS reaction (Figure 3B) was isolated, analyzed by NMR, and identified as ADIC,[7] which has been described previously as a precursor of ADC synthesis of the mutated *E. coli* ADCS K274A (Figure 3C).[4] To elucidate whether ADC and ADIC are synthesized by ADCS simultaneously or ADIC is a precursor of ADC, we incubated ADIC with *B. subtilis* ADCS. ADC was thus obtained and then converted to *p*ABA upon subsequent addition of ADCL (Figure 3F). Incubation of ADIC with ADCL alone did not yield *p*ABA, indicating that ADIC is not a substrate for ADCL.

With regard to the origin of the amino group of *p*ABA, two possibilities were considered: One involved intramolecular rearrangement of the ADIC amino group from position 2 to position 4, thus forming ADC via a [1,3]-sigmatropic shift. In this case, the amino group of *p*ABA would be identical to the one in ADIC. The other involved nucleophilic attack of an additional NH_3, provided by the glutamine amidotransferase PabA from glutamine, at position 4 of ADIC with concomitant loss of the C2 amino group, leading to formation of ADC. In this case the amino group of *p*ABA would originate from a second molecule of glutamine. To investigate the source of the *p*ABA amino group, we incubated isolated ADIC with *B. subtilis* ADCS and ADCL in [^{15}N] glutamine buffer. *p*ABA formed in this reaction was analyzed by LC−MS in order to determine whether the [^{15}N] amino group would be incorporated or not. Mass spectra showed unambiguously that only [^{15}N]-labeled *p*ABA (*m*/*z* 139) was formed, with no detectable traces of unlabeled [^{14}N]-*p*ABA (*m*/*z* 138). This led to the conclusion that an additional NH_3 generated by the glutamine amidotransferase PabA from glutamine is the origin of the *p*ABA amino group and that no intramolecular shift of the ADIC amino group from position 2 to position 4 occurs.

Steady-state kinetic parameters for *B. subtilis* ADCS and *E. coli* ADCS were determined. The k_{cat} and K_M values (0.08 s^{-1} and 40 μM, respectively) for *E. coli* ADCS with NH_4^+ as the nitrogen source are in good agreement with values reported previously.[4,8] For *B. subtilis* ADCS, k_{cat} and K_M are 0.004 s^{-1} and 380 μM, respectively, when the nitrogen source is NH_4^+ and 0.005 s^{-1} and 420 μM, respectively, when the nitrogen source is Gln. Interestingly, for *B. subtilis* ADCS, k_{cat} and K_M are on the same order of magnitude independent of the nitrogen source.

The present work represents a novel variation of the unified catalytic mechanism for chorismate-utilizing enzymes proposed by He et al.[4b] Therefore, we postulate that *p*ABA biosynthesis in *B. subtilis* proceeds in three steps (Figure 4B). First, ammonia is added at C2 of chorismate with concomitant loss of the C4 hydroxy group, yielding ADIC. The second step is the addition of a second molecule ammonia to C4 of ADIC with concomitant loss of the C2 amino group, yielding ADC. Both steps are catalyzed by ADCS. The final step is the ADCL-catalyzed elimination of pyruvate from ADC, which occurs identically in *E. coli* *p*ABA biosynthesis.

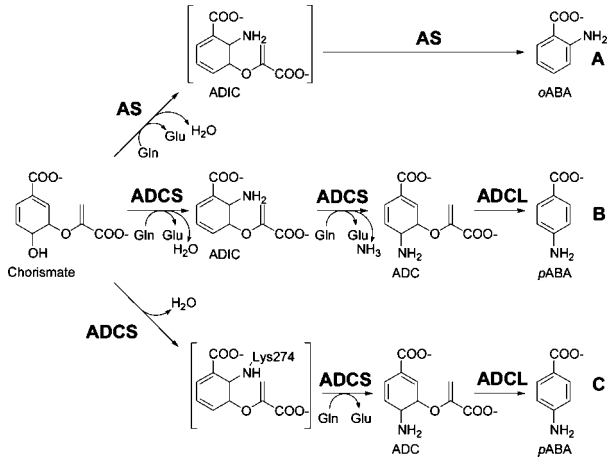

Figure 4. Comparison of three homologous chorismate-utilizing enzymes: (A) AS (TrpG + TrpE), (B) *B. subtilis* ADCS, and (C) *E. coli* ADCS. All three enzymes catalyze their reactions via addition of a nucleophile to C2 with concomitant loss of the C4 hydroxy group. In case of AS (A) and *B. subtilis* ADCS (B), the nucleophile is NH_3, whereas for *E. coli* ADCS (C), the ε-amino group of Lys274 is the nucleophile.

In comparison with the catalytic mechanism of *E. coli* *p*ABA biosynthesis, an additional glutamine is necessary for *p*ABA biosynthesis in *B. subtilis*. Formally, this glutamine can be reconstituted by the glutamine synthetase reaction from glutamate and NH_4^+ formed during *p*ABA biosynthesis:

$$\text{glutamate} + NH_4^+ + \text{ATP} \rightarrow \text{glutamine} + \text{ADP} + P_i \quad (1)$$

According to this reaction equation, *p*ABA biosynthesis in *B. subtilis* proceeds at the expense of one molecule of ATP, which is hydrolyzed to ADP and P_i.

Folic acid and tryptophan biosynthesis are closely linked on a genetic level in *B. subtilis*. Strikingly, in *B. subtilis*, the folic acid operon contains an amphibolic glutamine amidotransferase PabA/TrpG required for biosynthesis of both *p*-aminobenzoic acid and anthranilate.[9] The translation of the tryptophan operon as well as the translation of *pabA*/*trpG* is regulated by tryptophan-RNA-binding attenuation protein (TRAP). The gene for TRAP is encoded in the *mtrAB* operon, together with the gene encoding for GTP cyclohydrolase, which catalyzes the first step in folic acid biosynthesis.[10] Furthermore, PabB and AS subunit TrpE of *B. subtilis* show high sequence homology (see the Supporting Information), and all amino acids in the active site, including A283, are conserved. Furthermore, in view of the common reaction mechanism (i.e., nucleophilic addition of ammonia to C2 of chorismate), these findings reflect the close evolutionary relationship shared by the two *B. subtilis* enzymes.

To date, an ADIC synthase function has been shown for SgcD, a chorismate-utilizing enzyme required for biosynthesis of the enediyne antitumor antibiotic C-1027,[11] and proposed for PhzE,[12] which is required for phenazine biosynthesis. Except for these two secondary-metabolism enzymes and two mutant enzymes, the *E. coli* ADCS mutant K274A[4b] and the *Salmonella typhimurium* AS mutant H398M,[7] no enzymes with ADIC synthase function have been described to date. To our knowledge, *B. subtilis* ADCS is the first primary metabolism enzyme shown to exhibit ADIC synthase activity.

Sequence analysis of ADCS of various bacterial strains revealed that substitution of Lys with Ala in the active site is commonly found and that these *B. subtilis* PabB-like enzymes are phylogenetically related (see the Supporting Information). We therefore hypothesize that *p*ABA biosynthesis via ADIC as an intermediate is a general mechanism for all ADCS with lysine-to-alanine substitution in the active site.

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Supporting Information Available: Preparation of reagents and enzymes, kinetics of PabB, NMR data, and sequence and phylogenetic analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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